BINDING BEHAVIOR OF ANTITUMOR R,R-1,2-CYCLOHEXANEDIAMINE PLATINUM(II) WITH OLIGONUCLEOTIDES AND DNA

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Platinum modified oligonucleotides (5'-d(CpCpGpG)-3' and 5'-d(GpGpCpC)-3') and DNA were digested by endo- and exo-nuclease and the products digested were identified by means of HPLC. R,R-1,2-Cyclohexanediamine platinum(II) complex selectively binds to guanine residues to form intrastrand crosslink between two adjacent guanine bases through N(7)-N(7) sites.

Studies on the interaction of antitumor active platinum complexes with DNA and its constituents are now very interesting subject in relation with the mechanism of action.[1,2] Among the various platinum binding modes proposed, attention has been focused on the intrastrand platinum binding between two adjacent guanines.[2] Such a platinum binding mode has been proposed on the basis of H-NMR studies of Pt-modified oligonucleotides.[3-9] We will present an evidence for such an intrastrand crosslinking. In the previous paper, we reported that enzymatic digestion method using snake venom phosphodiesterase (VPD) and calf spleen phosphodiesterase (SPD) was useful for determination of platinum binding base in oligonucleotides.[10] In the present paper, the study has been developed to the determination of platinum binding bases in tetranucleotides. A preliminary results obtained for Pt-modified DNA will be also presented.

Reaction product of Pt(ox)(RR-dach)[11] with d(GpG) was examined. The reaction of Pt(ox)(RR-dach) with d(GpG) gave a single product, which was fractionated from HPLC with a weak cation exchange column (TSK-Gel 530 K), and it was characterized by UV difference spectroscopy. The UV spectrum of the product showed an absorption maximum at 260 nm, and the molar extinction coefficient per platinum was 22500 at 260 nm, indicating that the binding ratio between platinum and d(GpG) is 1 : 1.[12] The product did not show any UV spectral change in the pH 1.0 - 6.5, suggesting that N(7) of the guanine base, the proton attachment site, had already been occupied by the Pt(II).

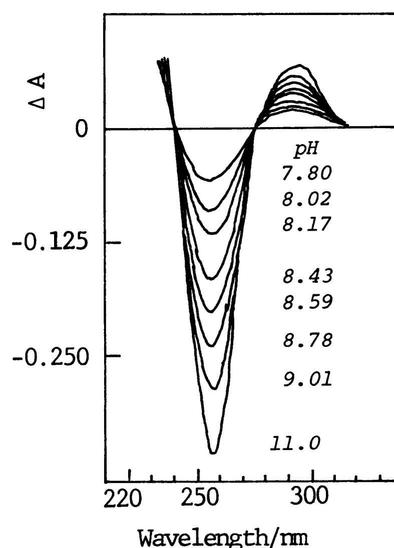

Fig. 1. Ultraviolet difference spectral change of Pt(RR-dach)(d(GpG)) as a function of pH.

Figure 1 shows the UV difference spectral change of the product as a function of pH, in which the reference cell contains the solution of the product at pH 4.6. The UV difference spectra showed a positive absorption maximum at 294 nm and a negative absorption maximum at 256 nm, and showed an isosbestic points at 240 and 266 nm.

This agrees with a characteristic pattern of UV difference spectrum arising from the deprotonated species at the N(1) of the N(7)-platinated guanosine, being reported in the previous paper.[13] The pKa value of the product, caculated from the relationship between ΔA and pH, was 8.5, a significantly lower value than that of the ligand (pKa = 9.6). Such a decrease of the pKa at the N(1) is characteristic of platinum binding to the N(7) site.[3-9,13] From the molar extinction coefficient, the UV difference spectral pattern, and pKa value at the N(1), it is concluded that the product is the complex with an interbase crosslink between two dG bases through N(7)-N(7). Although the product was treated with VPD and SPD, its phosphodiester bond could not be cut with these enzymes. This indirectly supports that the product is the complex with an interbase crosslink between two dG bases.

A 5'-d(CpCpGpG)-3' was allowed to react with an equivalent amount of Pt(ox)(RR-dach) at 37 °C for 5 d in aqueous solution Separation by HPLC gave two peaks shown in Fig. 2-1. Unplatinated d(CpCpGpG), which appeared at t_R = 3.0 min, was hardly retained by the column used, being expected from its negative charge and molecular size. The peak at t_R = 4.6 min is thought to arise from the reaction product because its peak height increased with incubation time. When the reaction mixture was treated with SPD and alkaline phosphatase (AP),[10,14] the peak height of the platinated d(CpCpGpG) (t_R = 4.6 min) decreased with incubation time and completely disappeared after 6 h incubation. The decrease in the platinated d(CpCpGpG) was accompanied with increasing two peaks at t_R = 5.2 and 6.4 min (Fig.2-2). The peak at t_R = 5.2 min was identical with dC in retention time. The peak height at t_R = 6.4 min reaches at a maximum after 4 h incubation and thereafter turns to decrease. With 2 d incubation, it disappeared completely.[15] The peak at t_R = 6.4 min appears to be a partially digested product, probably platinated d(CpGpG), because the decrease of the peak under consideration is accompanied with increasing peaks of dC and Pt(RR-dach)(d(GpG)) (Fig. 2-3). Eventually, enzymatic digestion products of the reaction mixture gave three peaks, t_R = 4.2 (dG), t_R = 5.2 (dC) and t_R = 10.6 (Pt(RR-dach)(d(GpG))). It is noteworthy that the peak area of dG is a marked small when compared with that of dC. The peak area of dC was approximately 90% of the original peak area which was obtained by the SPD and AP digestion of unplatinated d(CpCpGpG). On the contrary, the peak area of dG was only less than 20% of the original peak area. This strongly suggests that $Pt(RR\text{-}dach)^{2+}$ selectively binds to the guanine bases in d(CpCpGpG). This is finally supported by the identification of the peak of Pt(RR-dach)(d(GpG)).

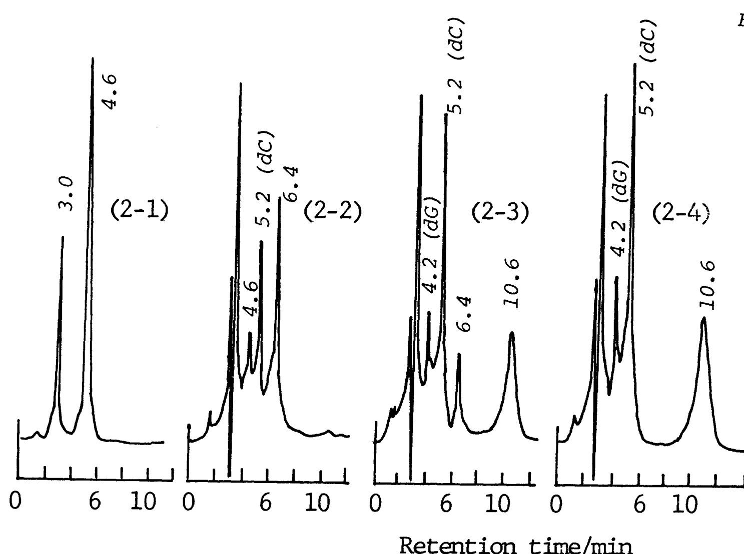

Fig. 2. HPLC chromatograms of the reaction mixture of Pt(ox)(RR-dach) with d(CpCpGpG) and enzymatically digested products.

2-1 ; Reaction mixture
2-2 ; Digestion products (2 h incubation with SPD and AP)
2-3 ; Digestion products (1 d incubation with SPD and AP)
2-4 ; Digestion products (2 d incubation with SPD and AP)

HPLC conditions

Column ; Toyo Soda TSK Gel IEX 530 K (weak cation exchange column)
Detector ; UV at 260 nm
Eluant ; 0.1 mol dm^{-3} KH_2PO_4 (1.0 ml/min)

Similar experiment was carried out for the reaction mixture of Pt(ox)(RR-dach) with 5'-d(GpGpCpC)-3'. Treatment of the reaction mixture with VPD gave Pt(RR-dach)(d(GpG)), 5'-dCMP, and 5'-dGMP as digestion products. When the solution after VPD digestion was further treated with AP, the chromatogram obtained was identical with that of Fig. 2-4. These results indicate that $Pt(RR\text{-}dach)^{2+}$ selectively binds to the guanine bases in d(GpGpCpC). Consequently, Pt(ox)(RR-dach) reacts with d(GpGpCpC) or d(CpCpGpG) to form the complex with intrastrand crosslink between adjacent guanine bases through the N(7)-N(7) sites.

Calf thymus DNA was allowed to react with various concentration of Pt(ox)(RR-dach) in 0.01 mol dm^{-3} phosphate buffer, pH 7.3, containing 0.01 mol dm^{-3} NaCl at 37 °C for 5 d (**r** = base/Pt = 0.01 - 0.20). A 200 µl of the reaction solution was treated with 600 units of DNase I in the presence of $MgCl_2$ at 37 °C for 6 h. After 20 µl of 1.0 mol dm^{-3} Tris-HCl, pH 8.9, and 1 unit of VPD was added to the solution, it was incubated at 37 °C for 16 h. The solution was further treated with 5 units of AP at 37 °C for 1 h.[16] Figure 3-1 shows the chromatogram thus obtained. The peaks at t_R = 5.2, 6.2, 8.2 and 8.9 min were identical with dT, dG, dA, dC in retention time, respectively. The broad peak at t_R = 4.0 min, which emerges at the void volume of the column, is thought to arise from platinated oligonucleotides because digestion of DNA did not give such a peak. The peak at t_R = 4.0 min increases with increasing **r**. Platinated octanucleotide and decanucleotide also have such a retention time.[17] When the solution after treating with AP was further treated with SPD, the broad peak under consideration almost disappeared and a new peak appeared at t_R = 24.0 min, being identical with Pt(RR-dach)(d(GpG)) in retention time. The peak area at t_R = 24.0 min increases with increasing **r** until **r** = 0.05 and thereafter it becomes constant (Fig. 4-1). However, it decreases at **r** > 0.15, probably because of incomplete digestion. The nearest neighbor frequency of d(GpG) in calf thymus

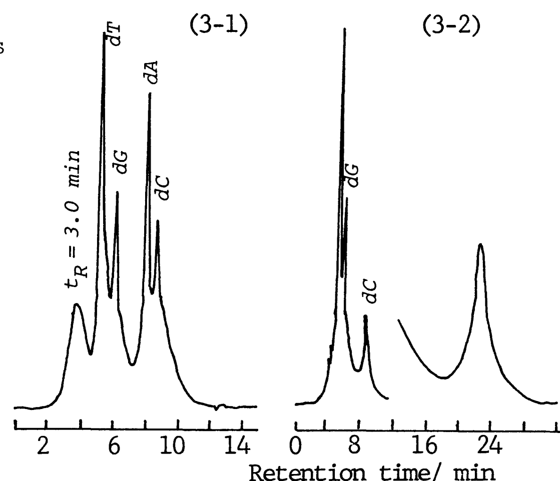

Fig. 3. HPLC chromatograms of enzymatically digested products of $Pt(RR\text{-}dach)^{2+}$ modified DNA.

3-1 ; Digested products being treated with DNase I, VPD, and AP
3-2 ; Digested products being treated with DNase I, VPD, AP, and SPD
Column ; Toyo Soda TSK Gel IEX 530 K (weak cation exchange column)
Detector ; UV at 260 nm
Eluant ; 0.05 mol dm^{-3} KH_2PO_4 (0.7 ml/min)

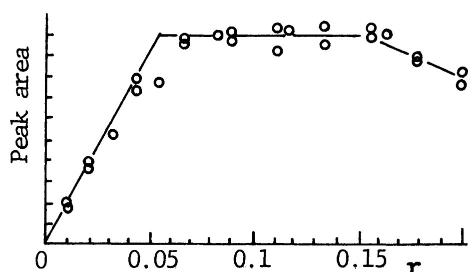

Fig. 4-1. Change of the peak area at t_R = 24.0 min, Pt(RR-dach)(d(GpG)), as a function of **r**.

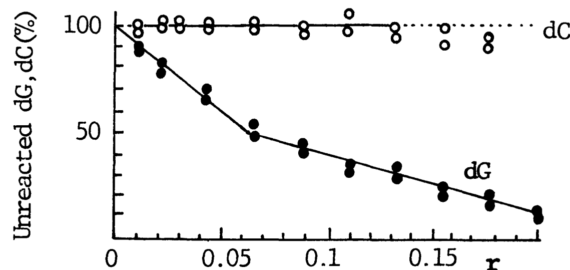

Fig. 4-2. Change of the unreacted dG and dC as a function of **r**.

DNA is about 5%. These results suggest that predominant attack of $Pt(RR\text{-}dach)^{2+}$ occurs at the adjacent guanine residues in DNA. Figure 4-2 shows a plots of unreacted dG and dC vs. **r**. Interestingly, the peak area of dC did not decrease in $\mathbf{r} < 0.15$, suggesting that there was no binding of $Pt(RR\text{-}dach)^{2+}$ with dC residue. The curve obtained from unreacted dG residue is divided for convenience to two straight lines. The slope of the line at $\mathbf{r} < 0.06$ is almost twice that at $\mathbf{r} > 0.06$. This suggests that $Pt(RR\text{-}dach)^{2+}$ selectively binds to dG residues in DNA, especially at low level of **r**. From these results, it is concluded that the preferred platinum binding occurs at N(7)-N(7) sites of the adjacent guanine bases in DNA. The complexes other than Pt(RR-dach)(d(GpG)) are now under investigation.

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11) Abbreviation. Pt(ox)(RR-dach) ; Oxalato R,R-1,2-cyclohexanediamine platinum(II). The complex was first synthesized by Kidani et al. and was found to have an excellent antitumor activity. Y. Kidani, K. Inagaki, and S. Tsukagoshi, Gann, 67, 921 (1976). Y. Kidani, K. Inagaki, M. Iigo, A. Hoshi, and K. Kuretani, J. Med. Chem., 21, 1315 (1978). A d(GpG) was allowed to react with an equivalent amount of Pt(ox)(RR-dach) at 37 °C for 7 d in aqueous solution.
12) Platinum concentration was determined by AAS.
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14) Digestion by SPD and AP was carried out at pH 6.2 (see Ref. 10). The phosphate group could be removed by AP within 0.5 h under the conditions used.
15) Plots of each peak area vs. incubation time follow the degradation reaction via intermediate (Pt(RR-dach)(d(CpCpGpG)) $\longrightarrow$ Pt(RR-dach)(d(CpGpG)) $\longrightarrow$ Pt(RR-dach)(d(GpG))).
16) Alkaline phosphatase contains as an impurity deaminase, probably adenosine deaminase. Therefore, subsequent incubation with AP leads to disappear the peak of dA (see Fig. 3-2).
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